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N-[CARBAMOYL]-β-D(+)-GLUCOPYRANOSYLAMINE METABOLISM BY MIXED RUMEN MICROBIOTA

by

RICHARD NIGEL COLEMAN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled N -[CARBAMOYL]-β-d-GLUCOPYRANOSYLAMINE METABOLISM BY MIXED RUMEN MICROBIOTA

submitted by RICHARD NIGEL COLEMAN

in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Animal Biochemistry

ABSTRACT

N-[carbamoyl]-β-D(+)-glucopyranosylamine was chemically synthesized from glucose and urea and the purity of the product was assessed using descending paper and gel exclusion chromatographic techniques. During the chemical synthesis either ^{14}C-urea or U^{14}C-glucose was incorporated into the molecule. N-[carbamoyl]-β-D(+)-glucopyranosylamine was found to be stable in a moderately acidic solution.

Homogenized rumen contents caused production of ^{14}C-CO$_2$ from N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine but neither sterilized rumen contents or Jackbean urease brought about this degradation. Acetohydroxamic acid at a concentration of 80 mg/ml resulted in 80% inhibition of the urease activity of homogenized rumen contents caused a reduction of the ^{14}C-CO$_2$ production from N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine brought about by the rumen contents. A similar effect was not detected with U^{14}C-glucose or N-[carbamoyl]-β-D(+)-[U-^{14}C]-glucopyranosylamine as substrate. In the presence of acetohydroxamic acid degradation of N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine by homogenized rumen contents resulted in accumulation of ^{14}C in urea and in accumulation of a detectable quantity of urea.

N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine degradation activity was associated with the particulate fraction of rumen contents.

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mg	milligram
g	gram
kg	kilogram
ul	microlitre
ml	millilitre
l	litre
um	micrometre
mM	millimolar
M	molar
h	hour
ga	gauge
D	dextroratatory
AC	alternating current
C	degrees centrigrade
AHA	acetohydroxamic acid
NCG	N-[carbamoyl]-β-D(+)-glucopyranosylamine
NPN	non-protein nitrogen
BBGU	barley based glucosyl urea
VFA	volatile fatty acid

INTRODUCTION

Protein is one of the more expensive constituents of animal diets. In addition, the use of protein supplements for animal feeds theoretically places the animals in direct competition with humans for protein. However, ruminants possess the ability to use dietary non-protein nitrogen (NPN), such as ammonia or urea, to produce protein of high quality for human consumption. Also since ruminants can utilize cellulose as an energy source they can be fed fibrous plant material that may not otherwise be used for food. Cellulosic plant material is the major product of photosynthesis, hence, harvest of this material using ruminants will remain important in supplying food for humans.

Presently the most quantitatively important NPN supplement is urea (Chalupa, 1968) which at this time is relatively inexpensive to produce. Urea, when ingested by the ruminant, is rapidly hydrolyzed to ammonia and carbon dioxide but much of the ammonia will not be utilized by the rumen microbiota unless readily fermentable carbohydrate is available.

Recently, a novel slow ammonia releasing NPN supplement for ruminants was developed jointly between the University of Alberta and the Research Council of Alberta (Milligan et al., 1972). This supplement was produced from barley and urea and called Barley Based Glucosyl Urea (BBGU). The major component of this supplement was N-[carbamoyl]-β-D(+)-

glucopyranosylamine or N-Glucosyl Urea (Milligan et al., 1972). These workers found that this chemical, when fed to ruminants, gave a slower increase of ruminal ammonia concentration and a later occurrence of maximum ammonia concentration as compared to an isonitrogenous quantity of urea. These characteristics of N-[carbamoyl]-β-D(+)-glucopyranosylamine made it a potential NPN supplement for ruminants consuming poor quality roughage since cellulose fermentation in the rumen and microbial protein synthesis occur over a very prolonged period.

The object of this research was to gain an understanding of the route of degradation of N-[carbamoyl]-β-D(+)-glucopyranosylamine by mixed rumen microbiota. More specifically, it was intended to determine the bond that is initially cleaved in this molecule.

LITERATURE REVIEW

1. Rumen Nitrogen Metabolism.

Dietary protein entering the rumen may either leave the rumen unmodified or be metabolized by the rumen microbiota. Those undigested proteins leaving the rumen are usually of lower solubility and are therefore more resistant to bacterial attack (Hungate, 1966; El-Shazly, 1958). The digestion of the more soluble proteins by the microbiota yields amino acids and oligopeptides. Amino acids can either be used directly by some rumen bacteria (Hungate, 1966) or be deaminated to yield volatile fatty acids (VFA) and ammonia (Hungate, 1966).

Ammonia is of central importance in rumen nitrogen metabolism. Nolan et al. (1972) and Mathison and Milligan (1971) indicated that as much as 80% of the nitrogen incorporated into bacteria was derived from the ammonia pool and they assumed that the remaining 20% was derived from amino acids of plant proteins. However, because of recycling (that is, death of bacterial cells and turnover of their contents) as much as 30% of the ammonia pool is continously being recycled through the amino acid and protein pools (Nolan et al., 1972).

Ruminal ammonia may be absorbed into the portal vascular system, and after conversion in the liver to urea, can be returned to the rumen via the saliva or by transfer or diffusion across the rumen wall (Lewis, 1961; Houpt,

1959) or be excreted in the urine. It has been suggested that the transport of ammonia across the rumen wall may be passive, and controlled by mass action and pH as the rumen wall is more permeable to ammonia than to ammonium ion (Mooney and O'Donovan, 1970).

The progressive amination of α-ketoglutarate through glutamate to glutamine is thought to be one the major sites of ammonia fixation in rumen microorganisms (Milligan, 1970) whereas rumen epithelium appears capable of fixing ammonia by forming glutamine from glutamate (Hoshino et al., 1966).

All NPN supplements for ruminants are metabolized to ammonia during their degradation and really serve as a source of ammonia for microbial growth in the rumen. One of the major problems in using NPN for ruminants is the rate of evolution of ammonia from the parent compound. If the rate of evolution does not match microbial incorporation there will be ineffective use of the supplement and the possibility of limited microbial growth as a result of the lack of ammonia or there will be accumulation of excess ammonia followed by its absorbtion and resultant dangers of toxicity in the host (Chalupa, 1968).

Numerous experimental results have shown that rumen bacteria, especially cellulolytic bacteria, can utilize ammonia for the synthesis of nitrogen containing cell components (Bryant and Robinson, 1962; Hungate, 1966; Hungate, 1975). On a larger scale, experiments involving addition of ammonium propionate and ammonium lactate to

ruminant diets yielded results showing that all supplemental nitrogen for the animal could be provided by these salts with no obvious detrimental effects (Allen et al., 1972; Dutrow et al., 1974). Further, ammonia may be used to monitor nitrogenous transactions within the rumen when used in the form of ^{15}N-ammonium sulphate or ^{15}N-ammonium chloride (Mathison and Milligan, 1971; Nolan et al., 1976).

In addition to the microbial use of ammonia considerable amounts of ammonia are absorbed from the rumen contents by the host. Ammonia fixation may occur in the rumen wall through glutamic dehydrogenase and glutamine synthase in sheep fed urea for extended periods (Britton and McLaren. 1973). This phenomenon is considered to be a possible rumen detoxification mechanism (Milligan, 1970).

A. <u>Urea.</u>

Rumen microorganisms are continually provided with urea that is recycled to the rumen via the saliva and across the rumen epithelium (Mathison and Milligan, 1971; Nolan, 1975; Smith, 1975; Kennedy and Milligan, 1977; Hinderer and von Engelhardt, 1976; Nolan et al., 1976). The extent to which recycling occurs varies reciprocally with nitrogen availability of the feed (Kennedy and Milligan, 1977). This demonstrates both a nitrogen conservation mechanism and a mechanism for upgrading the nitrogen status of the cellulolytic bacteria, since most cellulolytic bacteria require ammonia (Hungate, 1966).

Ruminal hydrolysis of urea has been found to be carried

out by rumen microorganisms belonging to the genera *Lactobacilli*, *Staphylococcus*, *Streptococcus* and *Klebsiella* (Cook, 1972). All but the last genus are Gram positive and all are facultative anaerobes. Ureolytic species of *Staphylococcus* and *Micrococcus* have been found and it was concluded that the facultatively anaerobic Gram positive cocci are probably responsible for a large proportion of the ureolytic activity of rumen contents (Cook, 1976). One highly probable reason that free living ureolytic bacteria in the rumen fluid are usually less than 1% of the population (Cook, 1972) is that they may be concentrated at or near the rumen wall. McGowan et al. (1978) have shown using both scanning and transmission electron microscopy that large numbers of Gram positive bacteria appear intimately associated with the rumen epithelium. They also suggest that the adherent bacterial population may differ in composition from those free in rumen fluid. Recently bacteria attached to the rumen wall have been implicated with ureolytic activity and the suggestion was made that rumen fluid urease represents the ureolytic bacteria sloughed from the surface (Cheng, 1978). These observations may account for the low numbers of ureolytic bacteria found free in the rumen (Cook, 1972).

2. Rumen Bacterial Cellulose Metabolism in Relation to Available Nitrogen.

Many rumen bacteria obligately require ammonia whereas others show a preference for it and still others require

preformed nitrogenous compounds (Hungate, 1966). Because of its role in rumen microbial metabolism ammonia has been shown to positively influence *in vitro* rumen fermentation rates (Mehrez et al., 1977). In grazing situations the ruminant can suffer from nitrogen depravation when some grasses translocate much of their nitrogen to their roots during dry periods (Schwartz and Gilchrist, 1975) or late in the season. Since ruminants derive much of their protein requirement by digestion of microbes that incorporated ammonia it would appear logical to augment their nitrogen status with NPN when additives are required (Hungate, 1966).

Carbohydrates supply most of the carbon and energy for rumen bacteria (Hungate, 1966). The carbohydrates usually comprise three classes; soluble monomeric units, soluble polymeric units and insoluble polymeric units. Soluble carbohydrates usually consist of starchs and pentosans which, because of their solubility, are easily digested. The insoluble cellulose is more refractory to digestion. Slowness of cellulose digestion is attributed to the molecular arrangement in which glucose is linked β-1,4 to form a linear chain. Each chain exhibits intra-chain hydrogen bonding mainly through the hydroxyl group on carbon three of the pyranose ring in one chain and the oxygen on carbon five in a parallel chain (Ranby, 1969). Intrachain bonding excludes water from the polymer and therefore solutes such as enzymes cannot gain access to their substrates and degradation is slowed.

It is thought that cellulase activity involves firstly a hydration step to give linear polymers (C1 activity) followed by hydrolytic cleavage to yield cellobiose (CX activity) (King and Vessal, 1969). This sequence appears consistant with those observations of rumen cellulose digestion where 3 to 5 h of contact of bacterial cell with substrate is required before digestion occurs (Hungate, 1966; Warner, 1965). To further substantiate this latter point, as much as 58.5% of cellulose ingested to the rumen may be eliminated by digesta passage as compared with only 15.3% removal of starch by this route (Maeng and Baldwin, 1975).

Since the initial steps of protein digestion to volatile fatty acids (VFA's) and ammonia are accomplished fairly rapidly (Hungate, 1966) and cellulose digestion is slow, the cellulose digesting bacteria will have mainly ammonia available as a nitrogen source. Hungate describes this phenomenon by stating:

"By the time the fiber-digesting bacteria have started growth, and during the period in which they attack the more resistant components of the forage, amino acids will be scarce. It is then not surprising that the fiber-digesting bacteria have the capacity to use ammonia as a source of nitrogen."

In this regard, any NPN supplement for ruminants consuming poor quality roughage should provide slow ammonia release in order for ammonia availability to coincide with fiber digestion and, hence, microbial incorporation of nitrogen and growth.

3. Slow Ammonia Releasing Non-Protein Nitrogen Feed Supplements.

Slow ammonia releasing NPN feed supplements supply ammonia to the rumen contents at a rate slower than that of urea. this usually means that ammonia is being released for longer than 1 h and often up to 5 h. this is done in an attempt to allow ammonia appearence to coincide with maximum cellulytic activity. A fast ammonia releasing NPN supplement, by contrast, will release its ammonia rapidly, often within the first h after ingestion. A good example of the latter is urea in which ammonia is rapidly formed usually within one half h post ingestion. The NPN sources discussed below are but a few of the potentially available sources for ruminant supplemental feeding.

A. Biuret

Biuret, formed by the pyrollysis of urea, has been promoted as a NPN supplement for ruminant diets. After ingestion biuret is degraded slowly in the rumen. In a biuret adapted sheep (adaptation period approximately 30 days) it was found that 35% of an 8.5 g dose of biuret passed from the rumen undegraded and that as much as 12% may

be found in the urine apparently undegraded (Fonnesbeck et al., 1975). Biuretase, the enzyme responsible for the cleavage of biuret to carbon dioxide, ammonia and urea, was found to be an intracellular bacterial enzyme (Fonnesbeck et al., 1975). A bacterium, as yet unnamed, that can degrade biuret has been isolated from sheep rumen contents (Bellingham and Bernstein, 1973). The microorganism was found to be a Gram positive non-sporeforming rod.

B. Nucleic Acids.

Nucleic acids may be considered a NPN supplement for ruminants but with the exception of poultry litter they are not fed (Fontenot and Webb, 1974). Hay may provide considerable intake of nucleic acids (McAllan and Smith, 1973). Nucleic acids are degraded slowly in the rumen resulting in a ruminal accumulation of thymine, hypoxanthine, uracil and xanthine from deoxyribonucleic acid and xanthine, hypoxanthine and uracil from ribonucleic acid (McAllan and Smith, 1973).

C. Ammonium Lignin Sulphonate.

A by product of the paper industry, ammonium lignin sulphonate, has been evaluated as an NPN supplement for ruminants. Results of this evaluation have shown that the rate of ammonia production was slower and availability of nitrogen equalled that of urea (Croyle et al., 1975).

D. Miscellaneous Amides.

Amides of acetic, propionic and butyric acids and crotonylidene and dicrotonylidene diureas have been assessed

as sources of supplemental slow ammonia releasing NPN for ruminants. The rates of ammonia accumulation in the rumen from these compounds were depressed when compared to an isonitrogenous quantity of urea (Atwal et al., 1971). After a 21 day adaption period of providing 3 g nitrogen/sheep/day the mixed amides resulted in only 67% of the ruminal ammonia concentration when compared with the ammonia produced from an isonitrogenous quantity of urea and the rate of decline in ammonia concentration was less for the former compounds.

E. Urease Inhibition.

Attempts have been made to slow the rate of hydrolysis of urea in the rumen by using exogenous urease inhibitors. Hydroxamic acids in general are known to inhibit *Proteus vulgaris* urease (Hase and Kobashi, 1967). This organism is not a normal rumen resident. However, when acetohydroxamic acid (AHA) was used at concentrations of 10 mM, 1 mM, 0.1 mM amd 0.01 mM in strained rumen fluid, urease was inhibited 84.2,23.6,5.5 and 0.0 percent, respectively, as compared to a control (Jones, 1968). The degradation of AHA also occurred and its disappearence followed a curvilinear course.

It was noted that AHA when used under *in vitro* culture conditions negatively influenced VFA production and the ratio of the VFA components (Jones, 1968). These changes may be artefacts due to the utilization of an inoculum of strained rumen fluid which does not contain the portion of the bacterial population attached to particulate rumen

material.

It has been concluded that hydroxamic acids inhibit *P. vulgaris* urease competitively and progressively (Hase and Kobashi, 1967). This mode of inhibition was further substantiated when rumen bacterial urease was found to be not only inhibited competitively but also reversibly by AHA (Mahadevan et al., 1977).

In vivo use of AHA on a short term feeding basis has been carried out with no apparent detrimental effects. AHA was found in the peripheral blood of lambs 1-3 h after they were fed the compound at a level of 0.1%-1.5% or less of their diet. No effect was detected in some of the more easily measured constituents such as rumen VFA's, rumen bacterial numbers and blood urea nitrogen (Jones and Milligan, 1975). Others have reported increased *in vivo* cellulose digestion due to the presence of AHA (Moore et al., 1968), presumably due to improved availablity of ammonia for the cellulolytic bacteria when they are growing.

Decreased activity of urease and prolonged optimal ammonia production from dietary urea due to ruminal addition of AHA have been reported; presumably this would aid the nitrogen nutrition of the cellulolytic bacteria (Jones and Milligan, 1975). Discounting some reported negative effects (Jones, 1968), which may to some degree be artefacts of *in vitro* culture, it appears that AHA can be used to inhibit rumen urease, at least for short periods of time (Moore et al., 1968; Jones and Milligan, 1975).

F. N-[carbamoyl]-β-D(+)-glucopyranosylamine.

In 1972 a carbohydrate adduct of glucose and urea was reported as possibly being an effective slow ammonia releasing NPN supplement for ruminants (Milligan et al., 1972). The compound described was called N-glucosyl urea or more properly N-[carbamoyl]-β-D(+)-glucopyranosylamine. There was found to be an adaptation period for degradation of N-[carbamoyl]-β-D(+)-glucopyranosylamine in the ruminant of about one week. The adaption was produced by feeding N-[carbamoyl]-β-D(+)-glucopyranosylamine to sheep twice daily. After adaption the initial rate of rumen ammonia accumulation was only 14-22% of that obtained from an isonitrogenous quantity of exogenous urea, and, the rate of decline in the rumen ammonia concentration after NPN treatment was slower (Milligan et al., 1972). During the adaptation period the ability of the rumen contents to produce ammonia from N-[carbamoyl]-β-D(+)-glucopyranosylamine increased from an initial low level to the final high level of 14-22%. Partial adaptation represents some intermediate ability of the rumen contents to produce ammonia from N-[carbamoyl]-β-D(+)-glucopyranosylamine. Others have shown that during *in vitro* experiments where rumen fluid was incubated with 1.25% (w/v) N-[carbamoyl]-β-D(+)-glucopyranosylamine, approximately 9% of the added substrate was not recoverable within 4 h whereas 75% of both added glucose and urea were not recoverable in the same time period (McAllan et al., 1975;

Smith et al., 1975). These workers also indicated that after 30 h of incubation about 50% of the N-[carbamoyl]-β-D(+)-glucopyranosylamine was not recoverable. However, these latter workers did not state how N-[carbamoyl]-β-D(+)-glucopyranosylamine was detected and whether the microbial population was supplied with other carbon, nitrogen and energy sources. In addition these authors used strained rumen fluid thus depriving the incubation mixtures of the attached bacterial population. Anaerobiosis was apparently not monitored and it is conceivable that facultatively anaerobic bacteria, when growing microaerophilically, could utilize N-[carbamoyl]-β-D(+)-glucopyranosylamine at a faster rate than under strictly anaerobic conditions (McAllan et al., 1975; Smith et al., 1975). In vitro incubation and cultural conditions will have to be standardized in order to obtain irrefutable data and reasonable conclusions.

Results from animal feeding trials have shown that N-[carbamoyl]-β-D(+)-glucopyranosylamine can support a portion of the ruminant's nitrogen requirements (Martin, 1976). Martin suggested that no significant difference existed in the rate of weight gain between sheep receiving either 10% BBGU, 20% BBGU, 50% BBGU or 10% soybean meal. The amount of the additives were as a percent of the total concentrate mixture. At 50 to 90% of the concentrate mixture acute ammonia toxicty was not encountered but after prolonged feeding, areas of demyelination occurred in the brain which may have been a result of a chronic excessive

absortion of ammonia. Martin also found that as the level of BBGU in the feed increased the blood urea nitrogen level increased and this is presumably as a result, in part, of high ammonia absorbtion. It was concluded from this study that BBGU or N-[carbamoyl]-β-D(+)-glucopyranosylamine was a potential slow ammonia releasing NPN supplement for ruminant diets but that its potential for future use will depend on production, marketing and promotional costs and the availability and costs of other forms of nitrogen (Martin, 1976).

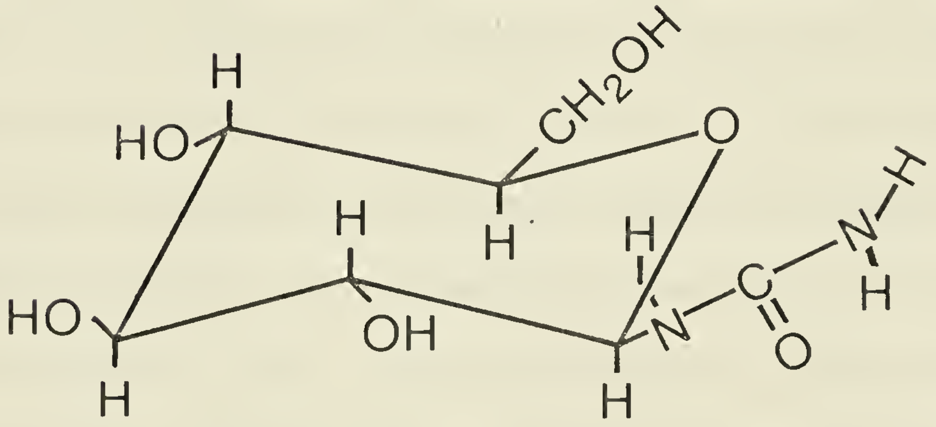
4. Chemical Synthesis of N-[carbamoyl]-β-D(+)-glucopyranosylamine.

N-[carbamoyl]-β-D(+)-glucopyranosylamine consists of a pyranose ring with an urea moiety attached through a β-glycosidic bond to carbon one of the glucose moiety (Goodman, 1958) (Figure 1). N-[carbamoyl]-β-D(+)-glucopyranosylamine was first synthesized in 1903 by Schoorl using an acid catalyzed condensation of d-glucose with urea (Schoorl, 1903). However, the yield of this compound was poor but in more recent times improved recoveries have been made (Hynd, 1926a; Hynd, 1926b; Benn and Jones, 1960). Another route of synthesis of N-[carbamoyl]-β-D(+)-glucopyranosylamine has been described by Fischer (1914) and by Badawi et al. (1966). Both reports describe the route as starting with the formation of tetra-O-acetyl-bromoglucose as outlined by Haynes and Newth (1955) and sequentially adding an isocyanate group followed by the addition of an

ammonia group. This method, however, requires strict anhydrous conditions which are difficult to attain and maintain.

FIGURE 1

A POSSIBLE CONFORMATION OF N-[CARBAMOYL]-β-D(+)-GLUCOPYRANOSYLAMINE



MATERIALS AND METHODS

1. Chemical Synthesis, Purification and Acid Stability of N-[carbamoyl]-β-D(+)-glucopyranosylamine.

A. Chemical Synthesis.

The procedure of Benn and Jones (1960) was used for the chemical synthesis of N-[carbamoyl]-β-D(+)-glucopyranosylamine with slight modification as shown in the following paragraphs. Into an Erlenmeyer flask fitted with an air jacketed condenser 10 g each of glucose and urea and 5 ml of 5% (v/v) aqueous sulphuric acid were measured. The mixture was stirred at 70C for 18 h at which time a further 5 ml 5 % (v/v) sulphuric acid was added followed by stirring for an additional 24 h. The mixture was poured into a beaker, allowed to cool and crystallize. Methanol was added to the crystals that had formed. The resulting mixture was filtered through Whatman 1 filter paper in a Buchner filter funnel. The filtered material was air dried, ground to a fine powder and placed in boiling methanol. Since urea is more soluble in boiling methanol than N-[carbamoyl]-β-D(+)-glucopyranosylamine the boiling methanol suspension was filtered through Whatman 1 paper in a Buchner funnel heated to 70C. This hot filtration process was repeated seven times and the retained material air dried and stored in plastic vials. Using this method glucose and/or urea labelled with

radioactivity [1] was incorporated into N-[carbamoyl]-β-D(+)-glucopyranosylamine.

B. Product Purity.

i. Descending Paper Chromatography.

Urea, glucose and N-[carbamoyl]-β-D(+)-glucopyranosylamine (a gift generously supplied by the Research Council of Alberta) were applied in approximately 5 mg amounts to the origin of Whatman 1 chromatography paper (23 x 57 cm). The sheet was placed in a descending paper chromatography tank containing n-butanol:pyridine:water (9:5:4) (v:v:v) both in the upper and lower chambers and developed for 16 h. The developed chromatogram was removed and air dried. Compounds were visualized and fixed by drawing the chromatogram through a solution of 0.1 ml saturated aqueous $AgNO_3$ dissolved in 20 ml acetone plus enough water to dissolve any precipitate that formed and then air dried. The chromatogram was sprayed with a solution of 20% (v/v) saturated aqueous NaOH in 95% (v/v) ethanol and dried. To enhance lighter areas the chromatogram was dried at 80-100 C. For fixation the chromatogram was passed quickly through 6M NH_4OH, washed in tap water until all base was removed and then air dried (Trevelyan et al., 1950).

ii. Sephadex G-10 Exclusion Chromatography.

A 100 x 2.5 cm glass column with flow adapters at both

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[1] The Radiochemical Centre, Amersham, England

ends was packed with a slurry of Sephadex G-10 [1] equilibrated in 10 mM KCl. The column was assembled and connected to a peristaltic pump with narrow bore tubing which in turn was connected to a three way valve. The column was washed with 10 mM KCl for 4 h in an ascending manner at a flow rate of 1 ml/min at which time the flow adapters were adjusted to the packed volume of G-10. One g N-[carbamoyl]-β-D(+)-[U-^{14}C]-glucopyranosylamine(specific radioactivity, 5.2x10^{4} dpm/g) and 1 g urea were dissolved in approximately 2 ml water and applied to the column via the three way valve. The column was eluted with 10 mM KCl and the eluate collected in 5.3 ml fractions. All fractions were assayed for urea and N-[carbamoyl]-β-D(+)-glucopyranosylamine using the descending paper chromatographic procedure, by detection of urea based on measurement of ammonia following Jackbean urease treatment and by assessing radioactivity from N-[carbamoyl]-β-D(+)-[U-^{14}C]-glucopyranosylamine. The method for the detection of urea was that of Fawcett and Scott (1960). For liquid scintillation counting of the N-[carbamoyl]-β-D(+)-[U-^{14}C]-glucopyranosylamine, 1 ml of each fraction was combined with 10 ml Aquasol 2 [1] and counted in a liquid scintillation spectrometer [2].

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[1] Pharmacia, Uppsala, Sweden.
[1] New England Nuclear, Mass, U.S.A.
[2] Mark 3, Searle Analytic, Ill, U.S.A.

C. Stability of N-[carbamoyl]-β-D(+)-glucopyranosylamine to Acid.

Concentrated acetic acid was diluted to 10% (v/v) with distilled water yielding a final pH of 2.2. To 200 ml of diluted acid 400 mg each of N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine(specific radioactivity, 5.35×10^3 dpm/20 mg) and N-[carbamoyl]-β-D(+)-[U-^{14}C]-glucopyranosylamine (specific radioactivity, 2.06×10^3 dpm/20mg) were added. The solution was mixed and dispensed in 10 ml aliquots to 20 Hungate tubes and sealed. The tubes were divided into two groups, one group was incubated at 21 C and the other at 39 C. Two tubes from each group were removed at 0,2,3,24 and 48 h, the CO_2 removed (Appendix 5) and 1 ml aliquots placed in 10 ml Aquasol 2. Both the CO_2 and 1 ml of the acidic solution were assayed for radioactivity in a liquid scintillation spectrometer.

2. The Effect of Using Sterilized Rumen Contents and Jackbean Urease on the Hydrolysis of Urea and on the Degradation of N-[carbamoyl]-β-D(+)-glucopyranosylamine.

Complex medium (Appendix 4) was made up to 60% of the final volume with water, rendered free of O_2 and equilibrated with CO_2. Following sterilization the medium was divided into two equal volumes (anaerobically under CO_2) and filter sterilized (pore size, 0.22 um [1]) N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine was added to one to a

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[1] Millipore Corp., Mass., U.S.A.

final concentration of 0.1% (w/v) and filter sterilized (pore size, 0.22 um) ^{14}C-urea was added to the other to a final concentration of 0.05% (w/v), both portions were mixed well. The two portions were each divided into four equal portions and inoculated with 40% (v/v) sterile (autoclaved) or non-sterile homogenized rumen fluid (Appendix 2), dispensed in 10 ml amounts into sterile Hungate tubes and capped under 100% CO_2. Jackbean urease [1](0.3 mg/tube, 141 su/mg) was added to the Hungate tubes as shown in Table 2. All tubes were incubated at 39 C for the required time (Table 2) then removed, acidified, the CO_2 trapped and the radioactivity counted in a liquid scintillation spectrometer (Appendix 5).

3. Effect of Acetohydroxamic Acid and Jackbean Urease on the Production of CO_2 from N-[carbamoyl]-β-D(+)-glucopyranosylamine by Mixed Rumen Microbiota.

Using complex medium (Appendix 4) and a 10% (v/v) inoculum of homogenized rumen contents (Appendix 2), the effect of AHA and Jackbean urease on the production of CO_2 from N-[carbamoyl]-β-D(+)-glucopyranosylamine was assessed.

Complex medium (Appendix 4) was made up to 90% of the final volume with water and autoclaved. Filter sterilized (pore size,0.22 um) N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine was added to give a final concentration of 0.1% (w/v) and the medium was divided into two equal

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[1] Worthington Biochemical Corp., N.J., U.S.A.

portions. Filter sterilized (pore size, 0.22 um) AHA was added to one portion to give a final concentration of 80 mg/100 ml. Ten percent homogenized rumen contents (Appendix 2) was added to both flasks, the contents mixed and transferred in 10 ml amounts aseptically and anaerobically to sterile Hungate tubes. The tubes from each flask were divided into two equal groups and incubated at 39 C. On the basis of an assumed AHA decay curve as described by Jones (1968) (Appendix 3), sterile AHA was added to the AHA group of Hungate tubes at between 4-8 h intervals during incubation in an attempt to maintain the AHA concentration at or near 80 mg/100 ml.

The final specific radioactivity of the medium was determined by oxidizing four aliquots of 1 ml each in a Biological Material Oxidizer [1], collecting the CO_2(Appendix 5) and assaying for radioactivity in a liquid scintillation spectrometer.

At timed intervals a tube was removed from each group before the next addition of AHA and 0.3 mg (141 SU/mg) of filtered sterilized (pore size, 0.22 um) Jackbean urease was added to one tube and not the other from the same treatment flask (with or without AHA). The urease containing tubes (two at each time interval) were incubated at 21 C for 1 h (see Table 3 for additions). Following incubation, the tubes were acidified and the CO_2 collected (Appendix 5) and

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[1] Beckman, Inc., U.S.A.

counted.

4. The Effect of Acetohydroxamic Acid on the Production of CO_2 from Glucose, Urea and N-[carbamoyl]-β-D(+)-glucopyranosylamine by Mixed Rumen Microbiota.

Complex medium was made up to 60% of the final volume with distilled water and sterilized. The medium was divided equally into eight separate sterile flasks. Duplicate additions were made to the flasks using filter sterilized (pore size, 0.22 um) aqueous solutions of: N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine, N-[carbamoyl]-β-D(+)-[U-^{14}C]-glucopyranosylamine, U^{14}C-glucose or ^{14}C-urea giving final concentrations of 0.1%, 0.1%,0.05% and 0.05% (all w/v), respectively. To one flask from each substrate group 8 mg filter sterilized AHA (pore size, 0.22 um) were added per 100 ml of medium; the other group served as a control. Each of the flasks was inoculated with 40% (v/v) homogenized rumen contents (Appendix 2), mixed well, 10 ml quantities dispensed aseptically and anaerobically into sterile Hungate tubes, sealed under 100% CO_2 atmosphere and incubated at 39 C. Following incubation the tubes were removed, acidified and the CO_2 collected and counted (Appendix 5). Sterile AHA was added at 4-8 h intervals to the remaining incubating tubes in an attempt to maintain the AHA concentration at or near 80 mg/100 ml (Appendix 3).

The specific radioactivity of the various media was determined by oxidizing duplicate 0.5 ml aliquots in a Biological Material Oxidizer and counting the $^{14}C-CO_2$.

5. Contribution to an Urea Pool by the Metabolites of N-[carbamoyl]-β-D(+)-glucopyranosylamine.

Complex medium (Appendix 4) was diluted with 60% of the required water and autoclaved. Filter sterilized (pore size, 0.22 um) N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine was added to yield 2% (w/v) (specific radioactivity, 2.5×10^2 dpm/mg N-[carbamoyl]-β-D(+)-glucopyranosylamine). After sterilization the medium was divided into two equal portions; to one was added filter sterilized (pore size, 0.22 um) AHA to a concentration of 200 mg/ml and none to the other. In preliminary experiments, AHA at a concentration of 100 mg/ml resulted in an accumulation of an urea pool that contained approximately 5% of the ^{14}C derived from N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine. Therefore, during this experiment AHA was used at a concentration of 200 mg/ml in an attempt to block urea degradation as completely as possible. The two separate flasks of medium were inoculated with 40% (v/v) homogenized rumen contents (Appendix 2), dispensed anaerobically and aseptically in 10 ml quantities into suitably marked sterile Hungate tubes, sealed under a 100% CO_2 gas phase and incubated in a water bath at 39 C. At intervals tubes were removed and from each tube duplicate 1 ml aliquots were applied in a line at the origin of two 23 x 57 cm sheets of Whatman 1 chromatography paper. Two ul of a reference containing 100 mg/ml each glucose, urea and N-[carbamoyl]-β-D(+)-glucopyranosylamine was applied at one edge of the chromatogram separate from the sample. The

chromatogram was developed as described previously (Materials and Methods). The edge of the chromatogram containing the reference was cut off, visualized and fixed using the $AgNO_3$ technique (Materials and Methods) and used as a location guide for the chromatogram. The areas of the latter coincident with the origin, N-[carbamoyl]-β-D(+)-glucopyranosylamine and urea were cut into very small peices and placed in 20 ml plastic liquid scintillation vials. To solublize the compounds, 1 ml of water was added to the vial and shaken by hand for 30 sec then 10 ml Aquasol 2was added and the vial shaken again and counted. The remaining 8 ml of medium in the Hungate tube was acidified and the CO_2 collected and counted (Appendix 5).

A similar procedure for visualization was followed for the second chromatogram. The urea containing area was cut out, the paper rolled, placed in a Hungate tube and 9 ml sodium phosphate buffer (0.6 mM, pH 6.6) (Fawcett and Scott, 1960) were added to solublize the urea. To each tube 1 ml Jackbean urease solution (0.3 mg/ml phosphate buffer (see previous sentence); 141 SU/mg) was added and the tube sealed. All tubes were incubated at 21 C for 1 h, acidified and the CO_2 collected (Appendix 5).

Controls were used to establish the efficiency of the chromatographic and collecting procedures. The three areas monitored were the collection of CO_2, the production of CO_2 from urea by Jackbean urease and the recovery of N-[carbamoyl]-β-D(+)-glucopyranosylamine from Whatman 1

chromatography paper after development.

To assess the recovery of CO_2, three 10 ml aliquots of $^{14}C-Na_2CO_3$ (5 mM/ml, 1.1 x 10^8 dpm/ml) were assayed for radioactivity. In addition, three identical aliquots were sealed in Hungate tubes, acidified to pH 1.5, the CO_2 collected (Appendix 5) and the radioactivity counted. Comparison of the two sets of data allowed assessment of the effectiveness of the procedure for the recovery of CO_2.

For assessment of N-[carbamoyl]-β-D(+)-glucopyranosylamine recovery from chromatograms 200 ul aliquots of N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine (specific radioactivity, 9.3 x 10^3 dpm/ml; 100 mg/ml) were applied in triplicate to Whatman 1 23 x 57 cm chrmatography paper along with a reference standard. The chromatogram was developed as outlined previously in Materials and Methods. The N-[carbamoyl]-β-D(+)-glucopyranosylamine areas were cut into small pieces and placed in plastic scintillation vials with 1 ml of water and shaken by hand for 30 sec to solublize the compound. Ten ml of Aquasol 2 were added to the vials and the radioactivity determined. Identical volumes of N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine solution were dispensed in triplicate directly into 1 ml of water and 10 ml Aquasol 2 and the radioactivity counted. Both sets of data were compared and the recovery achieved for the chromatographic procedure was calculated.

Using a method similar to the above, 0.1 ml of a ^{14}C-urea solution (specific radioactivity, 1.2 x 10^4 dpm/ml; 50

mg/ml) was applied in duplicate to Whatman 1 chromatography paper along with a reference urea solution. The chromatogram was developed as outlined previously (Materials and Methods). Urea areas were cut out and sealed in Hungate tubes. Ten ml sodium phosphate buffer (0.6 mM, pH 6.6) (Fawcett and Scott, 1960) containing 0.3 mg Jackbean urease were introduced through the stopper. The tubes were incubated at 21 C for 1 h, acidified, the CO_2 collected (Appendix 5) and counted. Three 0.1 ml aliquots of the same ^{14}C-urea solution were dispensed into Aquasol 2 and counted. Both sets of data were compared and the recovery for the chromatographic procedure calculated.

6. <u>The Location of N-[carbamoyl]-β-D(+)-glucopyranosylamine Cleavage Activity in Rumen Contents.</u>

The metabolism of N-[carbamoyl]-β-D(+)-glucopyranosylamine may occur in any of three possible locations in rumen contents. This activity may be extracellular, intracellular or cell associated.

Whole rumen contents were removed from a 70 kg wether that was receiving BBGU continuously for 6 months Appendix 6). A clean prewarmed (40 C) Dewar flask was filled with rumen contents and transported to the laboratory.

A series of filters including Whatman 50 paper, 8 um pore size membrane filter, 1.2 um pore size membrane filter and 0.2 um pore size membrane filter were assembled in separate holders and autoclaved. The filter units were transferred to a Freter anaerobic glove box (Aranki and

Freter, 1972) and connected aseptically in the above order. The rumen contents were transferred to the Freter box. After passing the rumen contents through four layers of cheese cloth they were placed in a sterile syringe. Cell-free rumen contents were obtained by forcing the strained rumen contents through the assembled series of filters and collected in a sterile Hungate tube.

A solution containing 100 mg/ml N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine(1.98 x 10^4 dpm/ml) and a solution containing 0.3 mg/ml Jackbean urease in sodium phosphate buffer (0.6 mM, pH6.6) (Fawcett and Scott, 1960) were each filter sterilized (pore size 0.22 um) and transferred to the Freter box.

The sterile rumen contents were dispensed aseptically in 2 ml aliquots to five sterile Hungate tubes. To each tube 0.1 ml of the sterile N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine solution was added. Two of these latter tubes recieved 1 ml of the urease solution and the other three tubes received 1 ml of sterile water. All tubes were sealed, removed from the Freter box and incubated at 39 C for four days. One ml aliquots were removed from all tubes and applied to a 27 x 53 cm sheet of Whatman 1 chromatography paper and co-chromatographed with a reference containing glucose, urea and N-[carbamoyl]-β-D(+)-glucopyranosylamine as previously described (Materials and Methods). The remaining 2.1 ml in the Hungate tube was acidified and the CO_2 collected (Appendix 5).

RESULTS AND DISCUSSION

1. Chemical Synthesis, Purification and Acid Stability of N-[carbamoyl]-β-D(+)-glucopyranosylamine

A. Descending Paper Chromatography.

This technique yielded Rf's calculated from the leading edge of the compound in question of 0.41, 0.32 and 0.16 for urea, glucose and N-[carbamoyl]-β-D(+)-glucopyranosylamine, respectively.

B. Sephadex G-10 Exclusion Chromatography.

N-[carbamoyl]-β-D(+)-glucopyranosylamine, as determined by descending paper chromatography, appeared first and was found to occur in fractions 36-50 inclusively followed by urea in fractions 50-70 inclusively (Figure 2). Recovery of N-[carbamoyl]-β-D(+)-glucopyranosylamine and urea from the exclusion chromatography was 105% and 102%, respectively. All preparations of N-[carbamoyl]-β-D(+)-glucopyranosylamine that were synthesized were examined using this technique; none were found to be less than 99.7% pure.

C. Stability of N-[carbamoyl]-β-D(+)-glucopyranosylamine to Acid.

The results in Table 1 are derived from duplicate and triplicate analyses with a range about the mean of less than 5%. These data show that there was virtually no degradation of N-[carbamoyl]-β-D(+)-glucopyranosylamine to CO_2; upon incubation for 45 h in 10% acetic acid, pH 2.2, recovery of label in CO_2 was never greater than 0.3%. All of the added

FIGURE 2

THE ELUTION PROFILE OF N-[CARBAMOYL]-β-D(+)-GLUCOPYRANOSYLAMINE and Urea from Sephadex G-10.

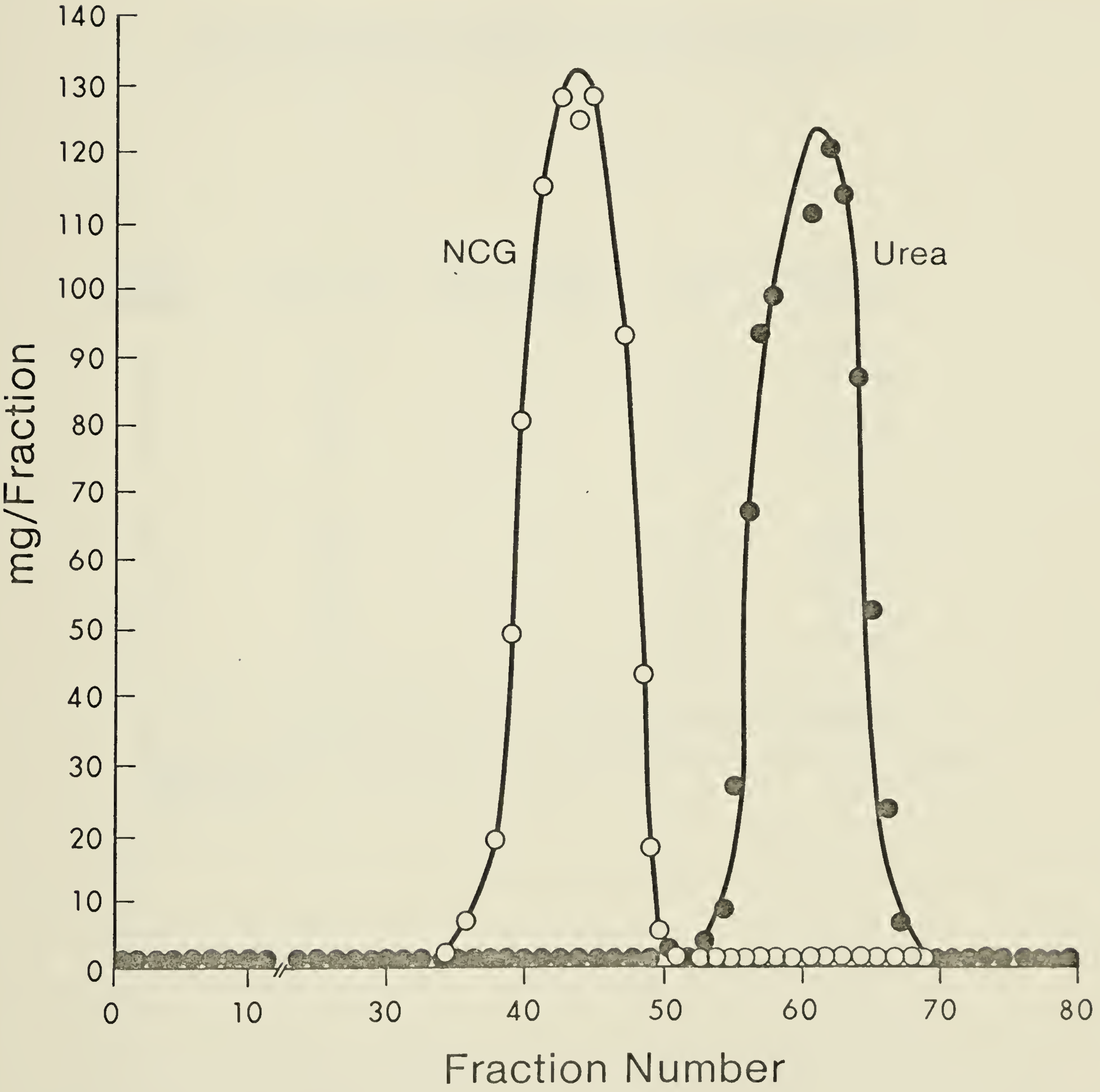

TABLE 1

THE EFFECT OF LOW pH ON THE PRODUCTION OF CO_2 FROM N-[CARBAMOYL]-β-D(+)-GLUCOPYRANOSYLAMINE[1]

TUBE NUMBER	INCUBATION TEMP. C	INCUBATION TIME-HOURS	% LABEL IN	
			CO_2	ACID
1	21	0	0.3	102.9
2	21	2	0.2	97.8
3	21	3	0.1	96.1
4	21	24	0.3	102.4
5	21	48	0.0	97.5
6	39	0	0.0	98.7
7	39	2	0.0	99.1
8	39	3	0.3	100.7
9	39	24	0.3	97.9
10	39	48	0.1	98.9

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[1] N-[^{14}C-CARBAMOYL]-β-D(+)-GLUCOPYRANOSYLAMINE

PERCENTAGES DERIVED FROM DUPLICATE AND TRIPLICATE ANALYSES

label was recovered in the acidic solution; no attempt was made to further identify the labelled material in the acidic solution.

2. The Effect of Using Sterilized Rumen Contents and Jack Bean Urease on the Hydrolysis of Urea and on the Degradation of N-[carbamoyl]-β-D(+)-glucopyranosylamine.

The data presented in Table 2 are derived from duplicate and single analyses and the range about the mean was less than 2%. Sterilized homogenized rumen fluid produced negligable levels of ^{14}C labelled CO_2 from either N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine or ^{14}C-urea. Jackbean urease, although clearly capable of catalyzing urea degradation did not result in production of ^{14}C-CO_2 from N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine. As shown by the initial samples for the first two urease treatments, this enzyme catalyzed very rapid hydrolysis of urea (14.5% and 11.8% recovery). The extent of degradation of ^{14}C-urea exposed to homogenized rumen contents was similar to that of ^{14}C-urea exposed to only sterlized rumen contents and urease. N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine was degraded slowly in the presence of homogenized rumen contents with the appearance of 11-12% of the original label in CO_2 in 48 h. These results bear out previous *in vivo* results where rumen ammonia was found to accumulate following rumen administration of 25 g of N-[carbamoyl]-β-

TABLE 2

BIOLOGICAL NATURE OF THE DEGADATION OF N[^{14}C-CARBAMOYL]-β-D(+)-GLUCOPYRANOSYLAMINE BY MIXED RUMEN MICROBIOTA

NCG[1] OR UREA[2]	UREASE[3]	INOCULUM[4]	INCUBATION TIME-H	RECOVERY OF LABEL-%[5]
UREA	+	S	0	14.5
UREA	+	S	12	84.7
UREA	+	V	0	11.8
UREA	+	V	1	86.8
UREA	–	S	0	0.1
UREA	–	S	12	1.6
UREA	–	V	0	7.7
UREA	–	V	1	74.7
NCG	+	S	0	0.4
NCG	+	S	12	0.5
NCG	+	S	48	0.4
NCG	+	V	0	0.5
NCG	+	V	12	4.7
NCG	+	V	48	10.9
NCG	–	S	0	0.1
NCG	–	S	12	0.1
NCG	–	S	48	0.2
NCG	–	V	0	0.5
NCG	–	V	12	4.1
NCG	–	V	48	11.8

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[1] N[^{14}C-CARBAMOYL]-β-D(+)-GLUCOPYRANOSYLAMINE-0.1% W/V

[2] ^{14}C-UREA - 0.05% W/V

[3] JACK BEAN UREASE - 0.3mg/10ml [141 SU/mg]

[4] 40% [V/V] HOMOGENIZED RUMEN FLUID - S=STERILE; V=VIABLE

[5] RECOVERY OF $^{14}CO_2$ DERIVED FROM NCG

INOCULUM FROM PARTIALLY ADAPTED SHEEP

PERCENTAGES DERIVED FROM DUPLICATE AND SINGLE ANALYSES

D(+)-glucopyranosylamine to sheep adapted to this compound (Milligan et al., 1972). The observation that urease plus viable inoculum did not yield greater amounts of CO_2 from N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine than the viable inoculum incubation indicates that a 40% (v/v) inoculum (in this case) supplied sufficient quantities of bacterial urease.

It can be concluded that viable rumen microbiota are required to produce urease to hydrolyze urea and to degrade N-[carbamoyl]-β-D(+)-glucopyranosylamine.

3. Effect of Acetohydroxamic Acid on the Production of CO_2 from N-[carbamoyl]-β-D(+)-glucopyranosylamine by Mixed Rumen Microbiota.

The data contained in Table 3 are the compilation of means from duplicate analyses and the range about the mean was less than 5%. The presence of AHA in the incubation lowers the total amount and the rate of production of CO_2 from N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine. The addition of exogenous Jack bean urease tended to result in appearence of more ^{14}C in CO_2 as compared to the amount in its absence. It will be recalled that it was previously found that incubation of N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine with only Jack bean urease did not yield $^{14}C-CO_2$.

As compared to the total label in CO_2 in Table 2 these results are higher. This is due to the use of rumen contents from a fully N-[carbamoyl]-β-D(+)-glucopyranosylamine

TABLE 3

EFFECT OF ACETOHYDROXAMIC ACID AND EXOGENOUS JACK BEAN UREASE ON THE PRODUCTION OF CO_2 FROM N-[CARBAMOYL]-β-D(+)-GLUCOPYRANOSYLAMINE[1]

		% RECOVERY OF ADDED LABEL AS CO_2 INCUBATION TIME - HOURS				
AHA[2]	UREASE[3]	0	18	29	42	70
-	-	2.4	17.4	ND	62.7	69.6
-	+	2.4	39.3	ND	66.7	79.8
+	-	1.3	6.6	5.6	21.7	38.7
+	+	0.7	6.5	12.4	27.3	42.9

[1] N-[^{14}C-CARBAMOYL]-β-D(+)-GLUCOPYRANOSYLAMINE-0.1%(W/V) IN CULTURE MEDIUM

[2] ACETOHYDROXAMIC ACID-MAINTAINED AT APPROXIMATELY 80 MG/100 ML OF MEDIUM

[3] JACKBEAN UREASE-0.3 MG/10 ML OF CULTURE MEDIUM(141 SU/MG)

INOCULUM FROM FULLY ADAPTED SHEEP

PERCENTAGES DERIVED FROM DUPLICATE ANALYSES

adapted sheep in the present case as compared to results from using rumen contents from a partially N-[carbamoyl]-β-D(+)-glucopyranosylamine adapted sheep in Table 2. From these data, then, it appears that urea may have been an intermediate in the degradation of N-[carbamoyl]-β-D(+)-glucopyranosylamine by mixed rumen microbiota.

4. The effect of Acetohydroxamic Acid on the Production of CO_2 from Glucose, Urea and N-[carbamoyl]-β-D(+)-glucopyranosylamine by Mixed Rumen Microbiota.

The results from this experiment are listed in Table 4. Each entry represents a mean value derived from either two or three replicates and the range about the mean was usually less than 4%. As can be readily seen AHA treatment produced 79% inhibition of CO_2 production from urea as compared to the control. Jones reported that AHA at 75 mg/100 ml produced about 80% inhibition of rumen urease (Jones, 1968). Thus there is good agreement between the present results and those of Jones (1968). AHA did not influence the production of CO_2 from glucose. When ^{14}C was present in the carbonyl carbon of the urea moiety of N-[carbamoyl]-β-D(+)-glucopyranosylamine, AHA reduced the rate of production and the total production of ^{14}C-CO_2 from N-[carbamoyl]-β-D(+)-glucopyranosylamine. When the glucose portion of N-[carbamoyl]-β-D(+)-glucopyranosylamine was labelled, AHA may have caused a slight initial depression from 10.7% appearance of label in ^{14}C-CO_2 to 6.8% appearance of label. It would be expected (Hungate,1966) that less than one third

TABLE 4

EFFECT OF ACETOHYDROXAMIC ACID ON THE PRODUCTION OF CO_2 FROM GLUCOSE, UREA AND N-[CARBAMOYL]-β-D(+)-GLUCOPYRANOSYLAMINE BY MIXED RUMEN MICROBIOTA

INCUBATION TIME-HRS	RECOVERY OF LABEL IN CO_2 FROM SUBSTRATES-% OF ORIGINAL ADDITION							
	GLUCOSE[1]		UREA[2]		NCG[3]		NCG[4]	
	-AHA	+AHA	-AHA	+AHA	-AHA	+AHA	-AHA	+AHA
0	0.2	0.2	1.2	0.2	0.9	0.8	0.3	0.4
4	3.5	4.6	73.4	11.5	12.0	7.9	ND	ND
7	9.2	14.5	98.1	19.0	21.5	8.2	ND	ND
18	14.8	13.1	ND	20.1	41.1	ND	ND	ND
24	ND[5]	ND	ND	ND	ND	ND	10.7	6.8
30	32.3	31.6	ND	18.9	98.5	65.2	ND	ND
46	36.8	36.8	ND	22.7	ND	ND	ND	ND
48	ND	ND	ND	ND	ND	ND	26.5	24.3

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[1] ^{14}UC-d-GLUCOSE-0.05%(W/V) IN CULTURE MEDIUM

[2] ^{14}C-UREA-0.05%(W/V) IN CULTURE MEDIUM

[3] N-[^{14}C-CARBAMOYL]-β-D(+)-GLUCOPYRANOSYLAMINE-0.1%(W/V) IN CULTURE MEDIUM

[4] N-[CARBAMOYL]-β-D(+)-[U^{14}C-GLUCOPYRANOSYLAMINE]-0.1%(W/V) IN CULTURE MEDIUM

[5] NOT DETERMINED OR CONTAMINATED

INOCULUM FROM FULLY ADAPTED SHEEP

PERCENTAGES DERIVED FROM DEUPLICATE AND TRIPLICATE ANALYSES

of glucose carbon would ultimately be oxidized to CO_2 during anaerobic rumen fermentation. Since the yield in this system was almost 37% at 46 h, the method of measurement may have trapped some labelled V.F.A.'s as well as CO_2; in this experiment no liquid trap was used but in subsequent experiments one was used. Thus AHA inhibited the urease activity of rumen microbes to approximately 80% and did not influence glucose conversion to CO_2. In addition, at 48 h AHA did not influence the conversion of the carbon of the glucose component of N-[carbamoyl]-β-D(+)-glucopyranosylamine to CO_2, but in the interval up to 30 h it did clearly inhibit the production of CO_2 from the urea moiety. Later results should be disregarded since in many tubes the resazurin indicated the tubes to be of high redox potential and probably contaminated with O_2. It would, therefore, appear that during its degradation by the rumen microbiota the urea component of N-[carbamoyl]-β-D(+)-glucopyranosylamine is released as free urea and is acted upon by microbial urease that was inhibited by AHA.

5. Contribution to an Urea Pool by the Metabolites of N-[carbamoyl]-β-D(+)-glucopyranosylamine

The CO_2 trapping apparatus was 99% efficient; the average radioactivity of the $^{14}C-Na_2CO_3$ was 1.06×10^5 dpm while the radioactivity found in the CO_2 produced from an equal amount of acidified $^{14}C-Na_2CO_3$ was 1.05×10^5 dpm. Both sets of data are means of triplicate samples. To correct for the CO_2 produced from the cultures a factor of 0.8 was used

since 2 ml of the total of 10 ml of culture medium were removed for paper chromatography. Therefore, the correction factor for the collection of CO_2 was 0.99, the correction factor for the volume loss was 0.8 and the overall correction factor was 0.99 x 0.8= 0.792. To obtain the corrected value for the CO_2 produced from cultures the following calculation was used;

dpm in CO_2 produced by cultures x 1/0.792.

Based on averages of duplicates only 714 dpm were found in CO_2 derived from 1235 dpm of ^{14}C-urea added to a chromatogram. Since the CO_2 collection system was 99% efficient, the correction factor became (714/1235) x 0.99=0.572. Further, only 1 ml out of the 10 ml of culture medium was sampled, therefore, the final factor was 0.1 x 0.572=0.0572. To obtain the corrected value for CO_2 derived from urea the following formula was used;

dpm in CO_2 from urea x 1/0.0572.

The inefficiency may have been due to inhibition or binding of the urease by the chromatography paper or adsorbtion of the urea by the paper causing the substrate to be unavailable to the enzyme. Adsorbtion of protein to cellulose has been observed by other workers (Lodish and Zinder, 1965). This being the case, higher levels of urea would have produced less of a fractional loss of the compound.

When 20 mg of N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine were chromatographed in duplicate and

the resulting spot was cut out and counted there was a yield of $3x10^3$ dpm. Equal quantities counted directly yielded $3.9x10^3$ dpm. Therefore, the recovery factor was $3x10^3/3.9 \times 10^3=0.769$ and since only 1 ml of 10 ml of medium was sampled the factor became 0.0769. Therefore, to determine the corrected value for dpm in N-[carbamoyl]-β-D(+)-glucopyranosylamine the following formula was used;

corrected dpm=dpm measured x 1/0.0769.

The results of this experiment were reported as per cent of the label added as N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine(Tables 5,6 and 7; Figures 3 and 4) that was recoverd in the isolated fractions.

The results in the tables were derived from either duplicate or single replicates and are expessed as the mean. In general the range about the mean was never greater than 6%. After the initial measurement, recovery of label was usually slightly less than the total theoretical quantity of label. The mean recovery for the label in the experiment including AHA was 95.8% while for the experiment excluding AHA was 94.2%. In both series there appears to be a consistent lack of recovery of 5-7% of the added label after the first sample. Methane biogenesis may account for a portion of this loss since CO_2 is a preferred substrate for most Methanogens (Mah, et al., 1977; Zeikus, 1977). The CO_2 trapping system used in this study would allow methane to pass out and be lost. However, the failure to account for only 5% of the added label in a complex system such as this

TABLE 5

THE DISTRIBUTION OF ^{14}C LABEL DERIVED FROM N-[^{14}C-CARBAMOYL]-β-D(+)-GLUCOPYRANOSYLAMINE IN CULTURES OF MIXED RUMEN MICROBIOTA IN THE ABSENCE OF ACETOHYDROXAMIC ACID.

	DISTRIBUTION OF ^{14}C-% OF ORIGINAL ADDITION				
INCUBATION TIME-HRS.	UREA[1] CO_2	CO_2[2]	NCG[3]	ORIGIN[4]	TOTAL RECOVERY[5]
0	0.3	0.1	100.7	0.2	101.7
19	0.1	12.5	75.5	0.5	89.3
28	0.1	16.4	73.4	0.5	90.8
45	0.8	22.2	73.6	0.8	97.8
65	0.1	30.2	62.8	0.4	94.0
89	0.1	36.2	57.1	0.7	94.5
115	0.7	44.0	49.9	1.5	96.4
137	0.7	47.2	38.9	2.0	89.1

[1] Urea band cut from chromatogram, reacted with urease, the CO_2 trapped and counted.

[2] CO_2 produced from culture.

[3] N-[^{14}C-CARBAMOYL]-β-D(+)-GLUCOPYRANOSYLAMINE-2%(w/v) in culture. Specific radioactivity=2.5 x 10^2 dpm/mg NCG.

[4] Origin of chromatogram.

[5] Urea CO_2 + CO_2 + NCG + Origin.

INOCULUM FROM FULLY ADAPTED SHEEP
PERCENTAGES DERIVED FROM DUPLICATE AND SINGLE ANALYSES

FIGURE 3

THE DISTRIBUTION OF ^{14}C LABEL DERIVED FROM
N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine(NCG)
IN MIXED CULTURES OF RUMEN MICROBIOTA
IN THE ABSENCE OF ACETOHYDROXAMIC ACID.

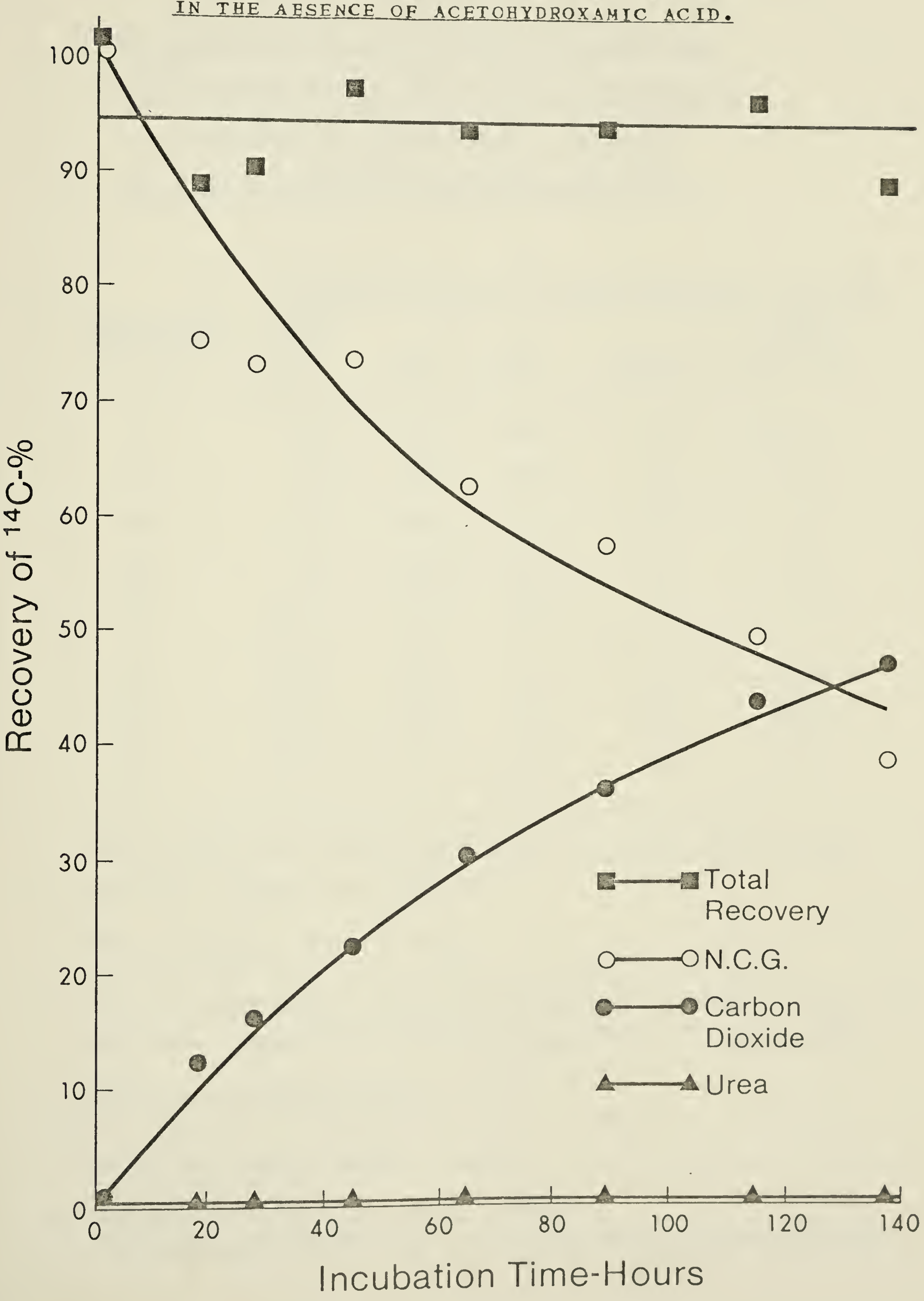

TABLE 6

THE DISTRIBUTION OF ^{14}C LABEL DERIVED FROM N-[^{14}C-CARBAMOYL]-β-D(+)-GLUCOPYRANOSYLAMINE IN CULTURES OF MIXED RUMEN MICROBIOTA IN THE PRESENCE OF ACETOHYDROXAMIC ACID.

INCUBATION TIME-HRS.	DISTRIBUTION OF ^{14}C-% OF ORIGINAL ADDITION				
	UREA[1] CO_2	CO_2[2]	NCG[3]	ORIGIN[4]	TOTAL RECOVERY[5]
0	0.7	0.1	101.9	0.2	102.9
19	2.2	8.3	80.2	0.8	91.5
28	4.0	15.1	75.8	1.6	96.5
45	7.4	16.9	63.5	1.5	89.3
65	11.2	23.2	59.7	0.9	95.0
89	16.9	22.9	51.9	0.7	92.4
115	21.7	23.0	50.1	1.3	96.1
137	32.2	25.4	44.3	1.4	103.3

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[1] Urea band cut from chromatogram, reacted with urease, the CO_2 trapped and counted.

[2] CO_2 produced from culture.

[3] N-[^{14}C-CARBAMOYL]-β-D-(+)-GLUCOPYRANOSYLAMINE-2%(w/v) in culture. Specific radioactivity=2.5×10^2 dpm/mg NCG.

[4] Origin of chromatogram.

[5] Urea CO_2 + CO_2 + NCG + Origin.

INOCULUM FROM FULLY ADAPTED SHEEP

PERCENTAGES DERIVED FROM DUPLICATE AND SINGLE ANALYSES

FIGURE 4

THE DISTRIBUTION OF ^{14}C LABEL DERIVED FROM N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine(NCG) IN MIXED CULTURES OF RUMEN MICROBIOTA IN THE PRESENCE OF ACETOHYDROXAMIC ACID.

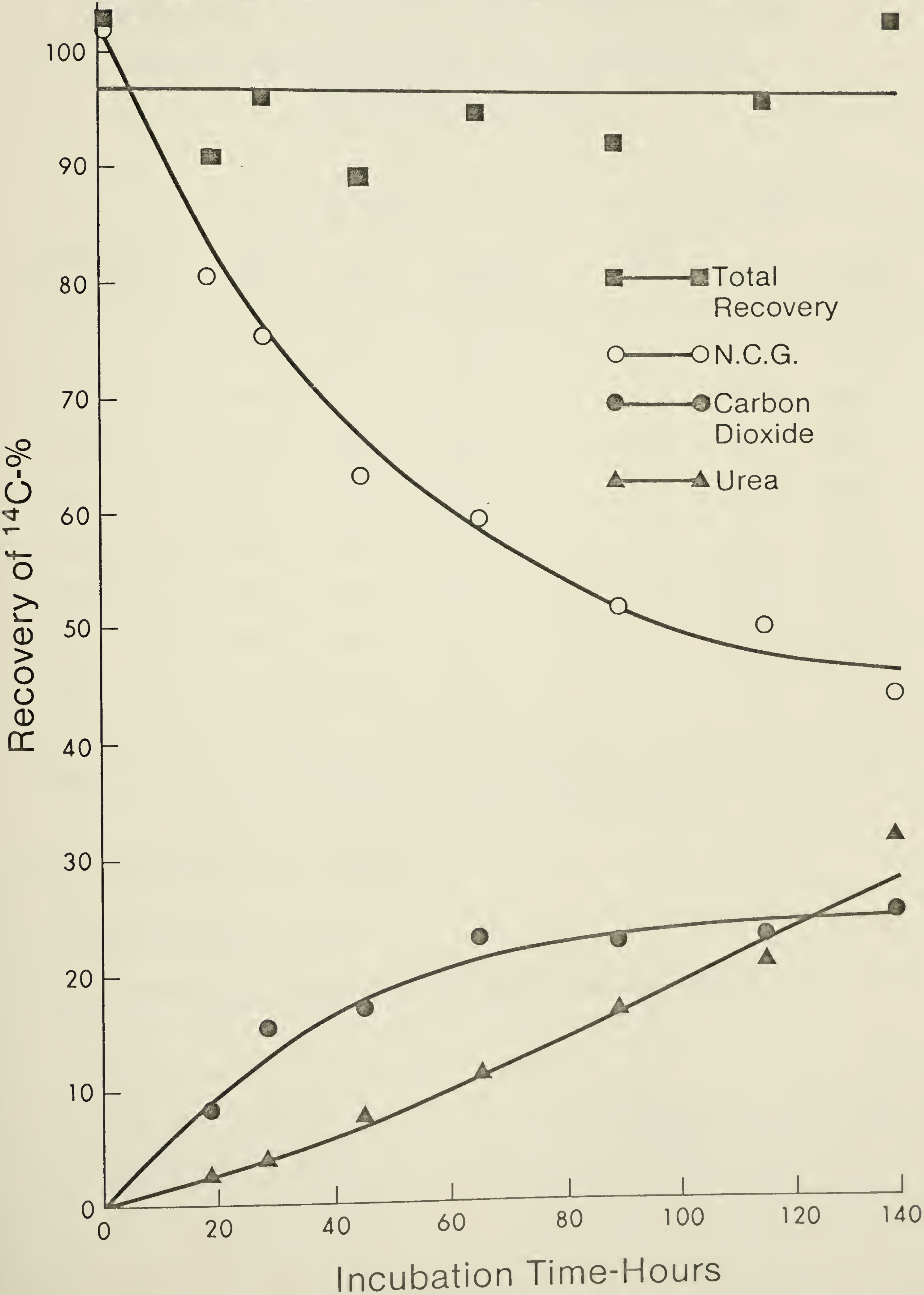

TABLE 7

COMPARISON OF RECOVERY OF LABEL FOUND IN CO_2 AND UREA IN ACETOHYDROXAMIC ACID INHIBITED AND UNINHIBITED CULTURES

	NO AHA			AHA		
INCUBATION TIME-HRS.	UREA[1] CO_2	CO_2[2]	TOTAL[3]	UREA[1] CO_2	CO_2[2]	TOTAL[3]
0	0.3	0.1	0.4	0.7	0.1	0.8
19	0.1	12.5	12.6	2.2	8.3	10.5
28	0.1	16.4	16.5	4.0	15.1	19.1
45	0.8	22.2	23.0	7.4	16.9	24.3
65	0.1	30.2	30.3	11.2	23.2	34.4
89	0.1	36.2	36.3	16.9	22.9	39.8
115	0.7	44.0	44.7	21.7	23.0	44.7
137	0.7	47.2	47.9	32.2	25.4	57.6

[1] UREA CO_2=Urea band cut from chromatogram and reacted with urease, the CO_2 trapped and counted.

[2] CO_2=Produced from culture.

[3] TOTAL=Urea CO_2 + CO_2.

[4] ACETOHYDROXAMIC ACID MAINTAINED AT 200 mg/100 ml.

was accepted as a limitation of the procedures used.

The inhibition of urease by AHA appears to increase with time as shown by a non-linear increase in label in urea and CO_2 (Table 6 and Figure 4). This effect may also be due to AHA accumulation in the medium following successive additions. However, Kobashi et al. (1962) have reported that the inhibition of urease by AHA increases with time, hence the phenomenon observed here may be an increasing effectiveness of AHA as an urease inhibitor. These two phenomena acting in concert may produce the AHA time effect.

A changed microbial population must be considered as a possibility resulting in changes in ability to metabolize N-[carbamoyl]-β-D(+)-glucopyranosylamine. Slight changes in a mixed population may be difficult, if not impossible, to monitor and no attempt was made to assess this aspect.

Results from this experiment clearly show that when the areas from paper chromotography of the medium that, by reference to a standard, contained urea were removed and subjected to the action of Jackbean urease, $^{14}C-CO_2$ was produced, indicating the presence of ^{14}C-urea. In addition, there was very much more label in urea in the cultures containing AHA than in the absence of AHA at all times after initiation of incubation (Tables 5,6 and 7;Figures 3 and 4). Since no more than 0.3% of the radioactivity of the N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine used in this study was due to urea contamination and the ^{14}C label was present only in the carbonyl carbon of the urea moiety of

N-[carbamoyl]-β-D(+)-glucopyranosylamine, the only reasonable conclusion was that the labelled urea detected in the cultures during incubation was derived as a result of metabolic conversion of the labelled N-[carbamoyl]-β-D(+)-glucopyranosylamine. The sum of the label in both urea and CO_2 in the control and the AHA treatment yielded values of similar magnitude even though the contribution of these two components differed widely (Table 7). As a final note in added proof of accumulation of urea in cultures in the presence of AHA, urea was detected using the $AgNO_3$ colour development method on a developed chromatogram from a sample of medium from a Hungate tube incubated for 45 h in the presence of AHA.

In view of the foregoing evidence it must be concluded that urea is an intermediate in the sequence of degradation of N-[carbamoyl]-β-D(+)-glucopyranosylamine by rumen microorganisms.

6. The Location of N-[carbamoyl]-β-D(+)-glucopyranosylamine Degradation Activity in Rumen Contents.

Using cell-free rumen contents from a sheep adapted to N-[carbamoyl]-β-D(+)-glucopyranosylamine, the recovery of the ^{14}C label in CO_2 in the tubes without added urease was 0.6% while the mean recovery of labelled CO_2 in the tubes to which Jack bean urease was added was 0.9%. Paper chromatographic analysis of the cell free rumen contents revealed no detectable qualitative changes in the amount of N-[carbamoyl]-β-D(+)-glucopyranosylamine present in any of

the tubes nor was there any indication of new products that might have arisen from degradation of N-[carbamoyl]-β-D(+)-glucopyranosylamine.

The initial metabolism of N-[carbamoyl]-β-D(+)-glucopyranosylamine apparently requires whole cells but it is yet to be determined if the location is intracellular or cell associated. The enzyme or enzymes may be thought of as being intracellular since no extracellular activity was found after ultra-filtration. If the enzyme (s) was cell-associated, low levels might be expected in the cell-free fluid as a result of some dissociation. It is also possible that enzyme activity was not found in the filtrate because of a very high affinity for the cellulose or cellulose acetate of the filters; the enzyme might have been retained on the filters. However, from the results of this experiment it appears that N-[carbamoyl]-β-D(+)-glucopyranosylamine degradation by the rumen microbiota requires the presence of microbial cells.

SUMMARY AND CONCLUSIONS

In summary, this project revealed that N-[carbamoyl]-β-D(+)-glucopyranosylamine can be chemically synthesized with incorporation of radioactive atoms into the molecule. Purification and analysis of the products of synthesis were accomplished. Gel exclusion chromatography was used to detect impurities in the products of chemical synthesis and using this technique N-[carbamoyl]-β-D(+)-glucopyranosylamine of high purity was obtained. Good agreement was found between the results of the latter technique and the results of a more conventional descending paper chromatographic technique of assesment of purity. In addition, N-[carbamoyl]-β-D(+)-glucopyranosylamine was found to be acid stable.

Jackbean urease rapidly hydrolyzed urea whereas the enzyme had no activity toward N-[carbamoyl]-β-D(+)-glucopyranosylamine. A sterilized inoculum of homogenized rumen contents did not result in production of $^{14}C-CO_2$ from N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine, whereas, a viable inoculum from the same source was active on the substrate and did produce $^{14}C-CO_2$.

AHA was found to inhibit rumen urease activity but was found not to influence production of $^{14}C-CO_2$ from $U^{14}C$-glucose or from N-[carbamoyl]-β-D(+)-[U-^{14}C]-glucopyranosylamine. When AHA was incorporated in the medium containing N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine and

incubated with homogenized rumen contents, from an adapted sheep, there was an accumulation of ^{14}C-urea. Urea was detected enzymically with Jackbean urease and chemically on a paper chromatogram after incubation. As well as appearing in urea, ^{14}C from N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine also appeared in CO_2 and the extent of appearence was reduced when AHA was added to the incubation medium.

In an attempt to locate the N-[carbamoyl]-β-D(+)-glucopyranosylamine degradative activity, whole rumen contents were filter sterilized and incubated with N-[^{14}C-carbamoyl]-β-D(+)-glucopyranosylamine. This resulted in no degradation that could be detected by either ^{14}C-CO_2 formation or by the formation of other metabolites as detected by descending paper chromatography.

It was concluded that N-[carbamoyl]-β-D(+)-glucopyranosylamine was metabolized by the rumen microbiota to urea and probably glucose if the cleavage is hydrolytic or to glucose-1-phosphate if the cleavage is phosphorolytic. The formation of glucose-1-phosphate would be more favourable since an adenosine triphosphate would not be expended for the phosphorylation. The cleavage of N-[carbamoyl]-β-D(+)-glucopyranosylamine requires microbial cells. Urease is not directly involved in the cleavage of N-[carbamoyl]-β-D(+)-glucopyranosylamine but is required in a secondary role for the hydrolysis of urea derived from N-[carbamoyl]-β-D(+)-glucopyranosylamine. It should be

emphasized that the degradation of N-[carbamoyl]-β-D(+)-glucopyranosylamine is a biological conversion.

The chemical bond that is being cleaved in N-[carbamoyl]-β-D(+)-glucopyranosylamine is novel when compared to chemical bonds known to be cleaved in biological conversions. Perhaps this uniqueness contributes to the slow degradation by rumen contents.

As a supplemental source of slow ammonia release NPN for ruminants, N-[carbamoyl]-β-D(+)-glucopyranosylamine may be one of the better compounds in this category. As shown by Martin (1976), the barley based N-[carbamoyl]-β-D(+)-glucopyranosylamine did not give signs of ammonia toxicity at substantial levels of feeding. It is consistent with the observation of slow degradation _in vivo_, that for _in vitro_ degradation lengthy periods of time were required. It is highly encouraging that the products of its degradation would be normal metabolites; indeed it should be noted that one of the sheep of this study was continuously fed BBGU for 18 months with no observable detrimental effects. Therefore, since the degradation of N-[carbamoyl]-β-D(+)-glucopyranosylamine is slow and the products are normal biolgical intermediates, N-[carbamoyl]-β-D(+)-glucopyranosylamine holds promise as a supplement for poor quality forage used as ruminant feedstuff.

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APPENDIX 1

Preparation of O_2-Free Gas.

The method of Hungate (1968) was followed but some minor changes were included. High grade H_2,N_2 and CO_2 gas cylinders were connected through regulators to a manifold. A 40 x 3 cm pyrex glass column was constructed with a single inlet, a T-shaped outlet and the interior packed with copper turnings before the T-shaped end was fashioned. Along the long axis and on the outside of the glass column were laid three 2 cm strips of asbestos tape around which was coiled 5.2 m of 16 ga nichrome wire making sure that the wire did not contact the glass. A 6 cm diameter collar of the 2 cm wide asbestos tape was made at both ends of the wire wrapped glass column and the entire assembly pushed into an outer glass sleeve such that the ends of the outer glass sleeve were sealed. The area between the sleeve and the glass column was a void space. Both ends of the nichrome wire were connected to an AC rheostat. A thermometer was sealed with a butyl rubbertubing sleeve in the arm of the T-shaped outlet in the longitudinal axis of the column. The thermometer continually monitored the 360 C operating temperature of the gas within the column. The other arm of the T-outlet was connected to a three-way manifold with butyl rubber tubing which in turn was connected to autoclavable 5 ml syringe bores that were packed with glass wool. Wintrobe needles

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

were connected to the syringe bores and used as gassing needles. All procedures requiring anaerobic conditions for this work included sparging with gas that was passed through this apparatus.

APPENDIX 2

Preparation of Homogenized Rumen Contents.

Since the contents of the ovine rumen are heterogenous in nature differing populations of microorganisms are likely to occupy the various biological niches. Fibrous particles will then support a microbial population adapted to the role of being attached to the fibre (Hungate, 1966;Schwartz and Gilchrist, 1975;Cheng et al., 1977;Mertens, 1977). Therefore, it was felt that the rumen contents used as an inoculum and the nutrient source should contain particulate material in additicn to the the fluid portion. To accomplish this, a portion of the rumen contents was removed from a permantly fistulated 50 kg whether using a 50 ml syringe and tube (ID 6 mm). The rumen contents were always removed 5 h after the morning feeding. The contents were strained through 1 layer of cheese cloth, to remove excessively large particles, and dispensed into a pre-warmed (40 C) clean Dewar flask. The flask was filled, stoppered and transported to the laboratory (taking 15-20 min). The stopper was then removed and any gas space was flushed with O_2-free CO_2 (Appendix 1), stoppered and stored at 39 C until use (usually within 30 min). For use the contents were transferred anaerobically and aseptically to a sterile

Omnimixer [1] vessel, the gas phase flushed with O_2-free CO_2 and sealed. The Omnimixer vessel was attached to the drive motor and operated at full speed for 2 min. This action reduced the particle size and homogenized the rumen contents so that they could be manipulated with a 10 ml serological pipette. This method of producing a suspension of rumen contents for inocula was used throughout this work.

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[1] Ivan Sorvall Inc., Conn., U.S.A.

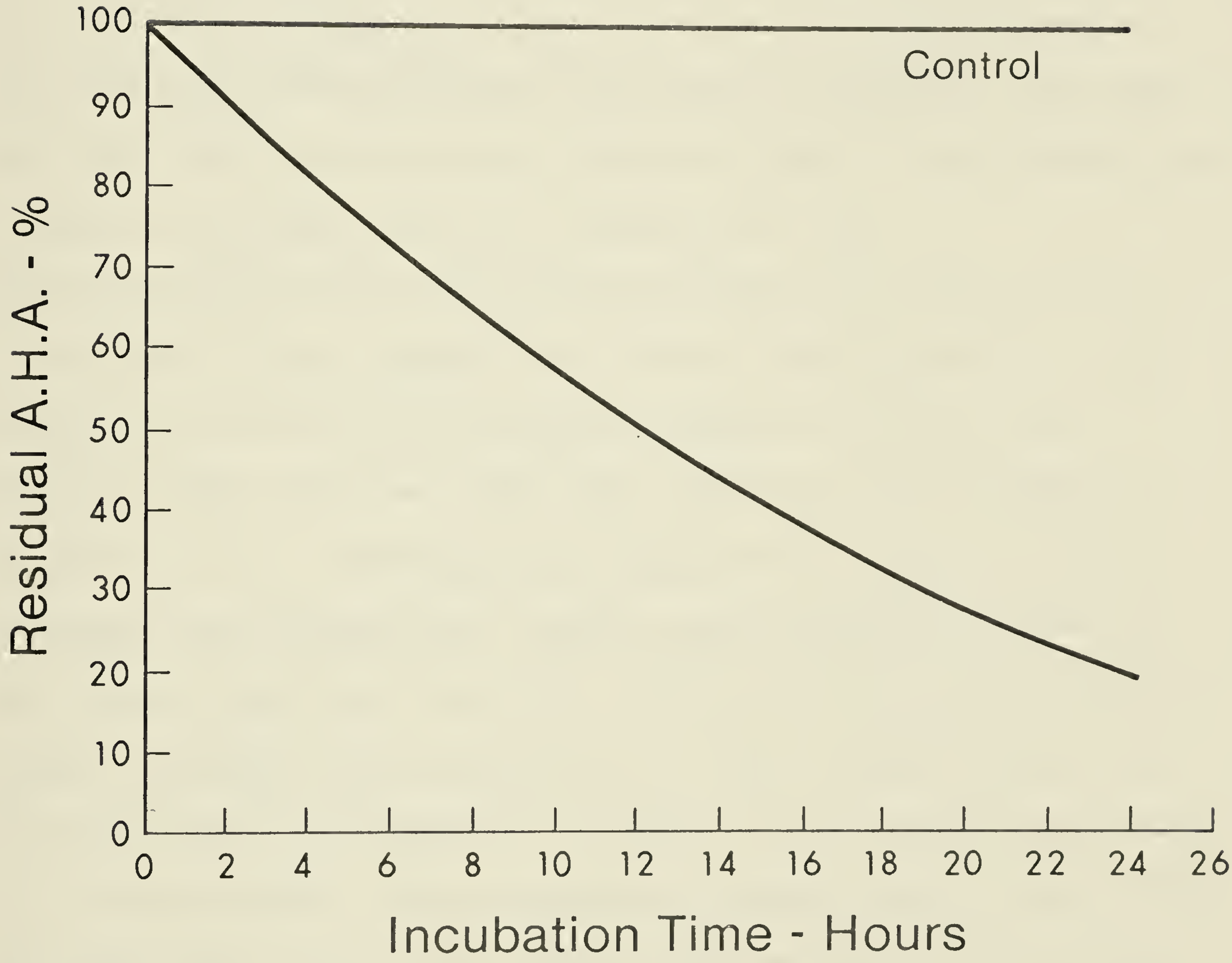
APPENDIX 3

Acetohydroxamic Acid Decay Curve.

AHA is metabolized by the rumen microbiota (Jones, 1968). In the investigation by Jones 0.09% glucose (w/v) was added to strained rumen fluid followed by an addition of AHA to result in a concentration of 5×10^{-3} M. Residual AHA was determined and plotted against time (Figure 5). This decay curve was used to determine the amount of AHA that should be added to cultures over time.

FIGURE 5

DISAPPEARENCE OF A.H.A. FROM STRAINED RUMEN FLUID CONTAINING GLUCOSE



after Jones (1968)

APPENDIX 4

Preparation of Complex Medium.

The complex medium used in this study differed slightly froma medium described by Caldwell and Bryant (1966). The two essential differences were that in the present medium 0.1-2.0% N-[carbamoyl]-β-D(+)-glucopyranosylamine was added and the CRF-2 of Bryant and Robinson (1961), was replaced by homogenized rumen contents (Appendix 2). The N-[carbamoyl]-β-D(+)-glucopyranosylamine was added to the sterilized complex medium as a filter sterilized (pore size 0.22 um) component. The complex medium of Caldwell and Bryant (1966) required 40% (v/v) clarified rumen fluid as a source of micro nutrients. In this study homogenized rumen contents were added after medium sterilization in place of the rumen fluid and were both a micro-nutrient source and a heavy inoculum. The composition of the complex medium used in this study is presented in Table 8. The medium was boiled in a thick-walled 250 ml Erlenmeyer flask, sealed under 100% CO_2 gas phase with butyl rubber stoppers, placed in a press and autoclaved for 15 min at 121 C. When required the rubber stopper was removed at the same time a sterile gassing needle was introduced into the neck of the flask to maintain 100% CO_2 gas phase. Filter sterilized additions, if required, were made at this time. In order to reduce the redox potential a few drops (less than 50 ul) of a filter

TABLE 8

THE COMPOSITION OF COMPLEX MEDIUM

COMPLEX MEDIUM

CONSTITUENT	QUANTITY
Homogenized rumen contents	40 ml
Glucose	0.05 g
Cellobiose	0.05 g
Soluble starch	0.05 g
NCG[1]	0.1 - 2.0 g
Cysteine·HCl·H_2O	0.025 g
Na_2CO_3	0.4 g
Resazurin	0.0001 g
Mineral solution	2.5 ml
H_2O	to 100 ml
CO_2 gas phase	100%
Final pH 6.6 - 6.8	

MINERAL SOLUTION

CONSTITUENT	QUANTITY
NaCl	1.72
$CaCl_2$	2.40
$MgSO_4 \cdot 7H_2O$	3.72
$(NH_4)_2\ SO_4$	17.95
H_2O	to 1 ℓ

DITHIONITE SOLUTION

CONSTITUENT	QUANTITY
$Na_2S_2O_4$	5 g
H_2O	to 100 ml

---

[1] NCG - N [CARBAMOYL] - β-D(+)-GLUCOPYRANOSYLAMINE

sterilized (pore size 0.22um) aqueous solution of $Na_2S_2O_4$ (50 mg/ml-see Table 8) were added. The resazurin indicator was reduced to its colourless form after this treatment indicating a redox potential of less than -0.043 Volts (Hungate, 1968). Using a CO_2 flushed serological pipette the medium was inoculated from the required source, mixed and dispensed into sterile Hungate tubes flushed with CO_2 and sealed. The tubes were then usually incubated at 39 C in a water bath.

APPENDIX 5

CO_2 Collection Procedure

Following incubation of the contents of the Hungate tubes, the pH was lowered to approximately 2.0 using concentrated H_2SO_4. As the acid was introduced (usually 0.3 ml/10 ml of medium) the pressure due to CO_2 production was released into a 22 ga needle inserted through the stopper which was connected to a collection system. The collection system consisted of a small liquid trap connected to a small H_2S gas scrubber filled with a solution of Cadmium acetate (15.25 g), Acetic acid (62.5 ml), Water (437.5 ml) connected to another gas scrubber containing 10 ml Ethanolamine liquid scintillation counting fluid (Toluene, 500 ml; Methanol, 300 ml; Monoethanolamine, 200 ml; P.P.O, 5.0 g; P.O.P.O.P., 0.2 g). After the pressure had equilibrated and while still connected to the system, the Hungate tube contents were heated to boiling. Another 22 ga needle connected to a source of N_2 was inserted through the stopper and the entire system flushed with the gas for 2 min. The scintillation counting fluid was then transferred to a 20 ml plastic vial along with the methanol washings from all internal surfaces of the last gas scrubber. Subsequently the vials were counted in a liquid scintillation spectrometer.

APPENDIX 6

Maintenance of Animals.

During the course of this study two Suffolk whethers were housed in quarters where the temperature varied between 17 and 23 C. One sheep weighed approximately 55 kg and the other approximately 50 kg. Both sheep were penned separately and maintained on 1200 g per day of bromegrass hay (Crude Protein-9.9%) divided into equal morning (0800 h) and nightly (1600 h) feedings. When the sheep's diets were to be supplemented with N-[carbamoyl]-β-D(+)-glucopyranosylamine, 100 g daily of BBGU were fed in equal portions morning and night. Water amd cobalt-iodized salt was allowed *ad libitum*.